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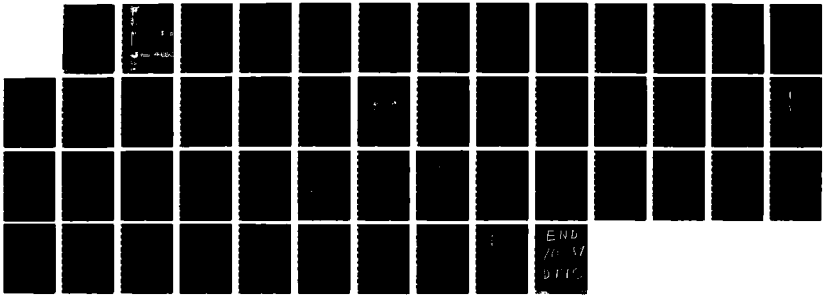

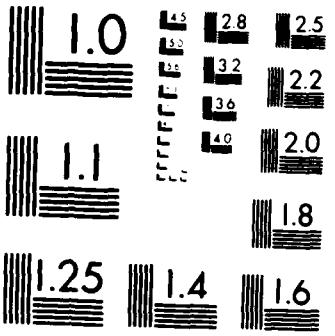

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AEROBIC BIODEGRADATION OF TRICHLOROETHYLENE

M.J.K. NELSON, P.H. PRITCHARD, S.O. MONTGOMERY, A.W. BOURGUIN

US ENVIRONMENTAL PROTECTION AGENCY
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Samples, suspected of having a capability to biologically transform trichloroethylene (TCE), were provided by Tyndall Air Force Base for verification and characterization of activity. Biological transformation of TCE was not observed in these samples. Other soil and water samples, obtained from the Pensacola area, were therefore screened for TCE degradation activity. One sample was found to have this ability and a gram-negative bacillus, which appeared to be responsible for the metabolic activity was isolated. The isolated organism degrades TCE (up to 3.4 μM) to less than 0.02 μM within 24 hours. TCE degradation occurred only when water from the original site of isolation and O_2 were in the medium. The isolate converted TCE into CO_2 and unidentified nonvolatile products. Phenol, toluene, o- and m-cresol were found to replace the site water requirement for TCE metabolism.

micro mole

ortho

meta

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EXECUTIVE SUMMARY

Trichloroethylene (TCE) is one of the most predominant and widely distributed groundwater contaminants. It is a major environmental problem because of its potential carcinogenicity and its stability in subsurface waters. This study was undertaken in an effort to discover microorganisms capable of degrading TCE to innocuous products. Isolation of a microorganism with TCE-degrading ability could lead to its eventual use for cleanup of TCE-contaminated sites.

Initial attempts to isolate TCE-degrading organisms from a TCE air stripping tower at Wurtsmith AFB were unsucessful. A variety of other water and sediment samples were then screened for the ability to degrade TCE. One of the water samples from an industrial waste treatment facility degraded detectable amounts of TCE. A bacterium, designated strain G4, that degraded TCE was isolated from this sample. Strain G4 only degraded TCE in the presence of water from the original site of isolation. The active component in the water was identified as phenol. Other aromatic compounds that could support TCE degradation were toluene, o-cresol, and m-cresol. The degradation could be prevented by inhibition of protein synthesis which suggested that the active component was inducing an enzyme necessary for TCE degradation. Phenol and toluene also induced degradative enzymes that cleaved the aromatic ring by meta-fission.

Strain G4 degraded TCE to carbon dioxide and nonvolatile products which were assimilated into cells. The degradation released all of the chlorine atoms of TCE in the form of chloride ions. The maximum rate of TCE degradation by strain G4 was 1.3 nmoles/minute/10^9 cells with the consumption of all TCE added (200 nmoles) within 3 hours. Strain G4 also dechlorinated two other volatile chlorinated aliphatic compounds, 1,1-dichloroethylene and cis-1,2-dichloroethylene.

Studies with several environmental water samples indicated that strain G4 could degrade TCE in natural waters. Furthermore, the natural microflora in the samples could be stimulated to degrade TCE by the addition of phenol or toluene. Two other pure cultures of bacteria, originally isolated for their ability to degrade aromatic compounds, also degraded TCE. A mutant of one of these bacteria, defective in the enzyme, toluene dioxygenase, was unable to degrade TCE. The results suggest that toluene dioxygenase was involved in the degradation of TCE.

This is the first report of the isolation of a pure culture capable of degrading TCE. The biodegradation appears to be the result of induction of an aromatic pathway used for the degradation of certain aromatic compounds. Other hazardous chlorinated aliphatic compounds may also be degraded by the same system. The ability of Strain G4 to convert TCE to harmless products indicates a strong potential for the use of biodegradation to decontaminate TCE-polluted environments. Treatment systems could utilize either pure cultures such as strain G4 in continuous bioreactors or natural microbial communities stimulated in situ by addition of the appropriate inducer.

We recommend continued research into the mechanisms of TCE degradation discovered by this research. The ability of strain G4 or similar isolates

to degrade other toxic halogenated compounds in addition to TCE should be examined to determine the range of this degradative ability. The enzymes involved and the mechanisms of detoxification of TCE should be determined to allow optimization of degradation of TCE as well as other halogenated compounds. Arrangement and regulation of the genes involved should also be studied. With further knowledge of the system, it will be possible to construct strains that degrade TCE and possibly other toxic halogenated compounds without the addition of aromatic inducers.

A system for onsite degradation of TCE either by use of a specific TCE-degrading strain or by stimulation of natural flora should be developed through laboratory bench-scale studies with expansion to a pilot-scale system to demonstrate cleanup of TCE at an actual contaminated site.

PREFACE

This report was prepared by the Microbial Ecology and Biotechnology branch of the U.S. Environmental Protection Agency Research Laboratory, Gulf Breeze FL 32561. The work was performed under Contract No. FY 8952-85-1008 for the U.S. Air Force, Headquarters Engineering and Services Center, Tyndall Air Force Base FL 32403-6001, between 1 January 1985 and September 1986. The HQ AFESC/RD project officers for this work were Captain Jack Jeter and Dr. Jim Spain.

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This report has been reviewed by the Public Affairs (PA) Office and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including Foreign Nationals.

This technical report has been reviewed and is approved for publication.

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SECTION I

INTRODUCTION

A. OBJECTIVE

Trichloroethylene (TCE) is volatile, nonflammable in air, and only slightly soluble in water. These characteristics make TCE a useful solvent as a spot remover, industrial metal cleaner, and degreaser for household, industrial, and military applications. Such extensive use of TCE in this regard probably explains its prominence as a groundwater contaminant (Reference 1). Several types of mechanical procedures have been used to reclaim TCE-contaminated groundwater. The expense of these procedures and their potential for causing secondary environmental effects have provoked a search for alternative methods. Biodegradation could be effective in removing TCE from groundwater through either in situ or aboveground treatment. Unfortunately, information on biodegradation of TCE is limited.

The objective of this project was to improve the information base on the aerobic biodegradation of TCE by the characterization of an aerobic bacterial culture that would metabolize TCE. Initial experiments were performed with samples provided by the U.S. Air Force, Tyndall, FL, from an aeration tower at Wurtsmith AFB, Michigan. Additional studies include the isolation and characterization of a bacterium from our own environmental samples that will completely mineralize TCE in the presence of aromatic compounds.

B. BACKGROUND

The presence of TCE in drinking water has caused a great deal of concern. Scientists have investigated different water treatment processes to decontaminate the groundwater. Although conventional water treatment is ineffective for removing TCE, aeration and adsorption to granular activated charcoal or Ambersorb® resin seem encouraging (Reference 1). Dilling et al. (Reference 2) measured evaporation rates of TCE during continuous stirring with a propeller attached to a 200 rpm motor. Fifty percent of the TCE evaporated from water in 19 minutes, while 90 percent evaporated after 63 minutes. They also measured the influence of NaCl, organic matter, and sunlight on evaporation rates. Sodium chloride (3.0 percent) decreased evaporation rates by 10 percent. The addition of peat moss (~ 500 ppm) initially accelerated TCE evaporation, but this loss was not sustained. Sunlight had the greatest effect. TCE disappeared when irradiated with long-wavelength light in the presence of nitric oxide or nitrogen dioxide. Most of the disappearance was probably caused by oxidation.

Wilson et al. (Reference 3) examined the degradation potential of microbes found in groundwater systems. TCE disappeared at the same slow rate in both autoclaved and nonautoclaved samples, indicating the loss was an abiotic process. However, Parsons et al. (Reference 4) conducted experiments that indicated biological activity was responsible for tetrachloroethene and TCE transformation in microcosms containing

cultured bacteria. Successive dehalogenations occurred from tetrachloroethene to trichloroethene and then to chloroethene, cis- and trans-1,2 dichloroethene and dichloromethane. These transformations did not occur in autoclaved controls. Wilson and Wilson (Reference 5) exposed unsaturated soil to natural gas and found that TCE was extensively mineralized to CO_2. Without exposure to natural gas there was no degradation of TCE.

Several studies suggest that TCE may be degraded under methanogenic conditions. Bouwer and McCarty (Reference 6) found that some chlorinated aliphatics in low concentrations were completely oxidized to carbon dioxide in continous-flow systems. Tetrachloroethylene was also partially mineralized to carbon dioxide (Reference 7). In these studies, TCE was the major product formed from tetrachloroethylene, but it was not established whether it was an intermediate in CO_2 production.

Researchers at Idaho National Engineering Laboratory in Idaho Falls, Idaho, investigated the possibility that TCE might be degraded by a biofilm which had accumulated in an air-stripping tower (Reference 8). Optimal growth was observed in 1 percent (wt/vol) glucose buffered basal salts medium, pH 6.0, temperature 35°C and ammonium sulfate as the nitrogen source. Bacteria with apparent TCE degradation potential were isolated from the air-stripping tower samples. One culture, designated Wurtsmith 3 (keyed to genus Citrobacter), grew under aerobic or anaerobic conditions, utilizing nitrogen in either organic or inorganic forms. The bacterium did not grow on methanol, ethanol, or acetate. In initial [^{14}C]TCE experiments, the Wurtsmith 3 cultures appeared to convert a portion of the TCE to CO_2. However, no $^{14}CO_2$ was produced in the active cultures when the experiments were repeated. It is not clear whether the original activity was due to biodegradation, which was subsequently lost, or to an artifact of the experiment.

Our report documents the isolation and characterization of an aerobic nonmethylotrophic bacterium which rapidly degrades TCE to CO_2. The existence of this organism improves the outlook for biological treatment of TCE-contaminated groundwater.

C. SCOPE/APPROACH

The approach of this project was to develop analytical and microbiological methods to enrich for an aerobic TCE-degrading organism from samples and isolates (Reference 8) supplied by the U.S. Air Force. In addition, with these methods, a variety of additional environmental samples with histories of TCE or other chlorinated organic compound contamination were screened for their potential to promote the degradation of TCE. Positive samples were screened for bacteria capable of TCE metabolism using standard microbiological procedures; metabolism of TCE by a pure culture was confirmed with growth and mineralization studies. Comparison of this information with the literature will indicate the potential of using this organism, or the process it catalyzes, to biologically treat TCE-contaminated groundwater.

SECTION II

ISOLATION OF A PURE CULTURE THAT DEGRADES TCE

A. METHODS

1. Batch Enrichment Systems for TCE-Degrading Microorganisms

Initial enrichment systems consisted of sealed 1000 mℓ Erlenmeyer flasks containing 250 mℓ of minimal salts broth (MSB, Table 1), TCE, and an environmental sample inoculum.

TABLE 1. MINIMAL SALTS BROTH (MSB) COMPOSITION

Major Ingredients	g/ℓ
Na_2HPO_4	5.68
KH_2PO_4	5.45
$(NH_4)_2SO_4$	3.0
$MgSO_4 \cdot 7H_2O$	0.289
Nitrilotriacetic acid disodium salt (NTA)	0.250
$CaCl_2$	0.067

Trace Metals and EDTA	mg/ℓ
Ethylenediamine tetraacetic acid disodium salt (EDTA)	2.5
$ZnSO_4 \cdot 7H_2O$	10.95
$FeSO_4 \cdot 7H_2O$	5.0
$MnSO_4 \cdot H_2O$	1.54
$CuSO_4 \cdot 5H_2O$	0.392
$Co(NO_3)_2 \cdot 6H_2O$	0.248
$Na_2B_4O_7 \cdot 10H_2O$	0.177

Flasks were incubated in an incubator fitted with an air purification apparatus (filtration through a special grade of charcoal designed to trap volatiles) to prevent TCE leakage from the rubber stoppers. Evidence for TCE degradation was derived by plating subsamples of the flask's contents on minimal salts agar. Colonies that grew in a TCE atmosphere

(desiccator jar with a small beaker of TCE) were presumptive TCE degraders. In other enrichment systems, TCE disappearance was monitored in samples removed from the headspace with a gastight syringe and analyzed by gas chromatography under conditions shown in Table 2.

TABLE 2. GAS CHROMATOGRAPHIC PARAMETERS FOR ANALYZING GAS SAMPLES CONTAINING TCE

Instrument: Hewlett Packard 5790

Column: Supelco Capillary SPB5 (30 m)

Detector: Electron capture

Oven temperatures: 60°C isothermal

Injector temperature: 100°C

Detector temperature: 325°C

Carrier gas: H_2, 1 mℓ/min

Makeup gas: Ar/CH_4-90/10, 45 mℓ/min

Septum purge splitless for 0.5 min after injection

Standard injection: 20 µℓ of gas sample with Hamilton gastight syringe

Each tube system contained 5 mℓ of sterile MSB, 500 µg TCE/mℓ, and 1 mℓ of inoculum. Soil samples were suspended in water (0.5 to 1 g/100 mℓ) for use as inocula. Sterile controls were prepared identically but were autoclaved for 15 minutes at 121°C before the addition of TCE. Undiluted water samples were used for inocula. These were amended with MSB by adding concentrated mineral stock solutions to yield the required concentrations. Inocula were obtained from the Air Force (Wurtsmith AFB air-stripping tower), the Pensacola Naval Air Station (NAS) industrial treatment plant (known to contain various chlorinated compounds), a hazardous waste site near Pensacola (soil contaminated with pentachlorophenol, cresote, and TCE), Escambia Treating Company (soil contaminated with pentachlorophenol), and an effluent holding pond at the EPA laboratory (known to have received many chlorinated pesticides for more than 15 years).

Other enrichments were performed using MSB supplemented with vitamins or yeast extract and the following potential co-substrates: acetate, succinate, benzoate (100 mM, each), phenol (10 mM), ethylene, methane (10 percent headspace) and fuel oil (0.002 percent vol/vol). Replicates of all enrichments were autoclaved as sterile controls. TCE disappearance in active samples after incubation, as compared to sterile controls (based on gas chromatographic peak area of TCE in headspace samples), was used to screen for TCE biodegradation.

2. Improved Experimental Systems for Measuring TCE Biodegradation

During the early work with active enrichment experiments variability among replicates was as much as ± 50 percent. It was believed that inadequate gas exchange, as well as incomplete mixing of the samples in the test tubes used for experiments, might be contributing to variability of results. A new system was therefore developed, using 50 mℓ serum vials (Wheaton) sealed with butyl rubber stoppers and containing 10 mℓ of MSB, one mℓ of inoculum, and 50 nmol of TCE. The bottles were incubated at 26°C on a gyrotory shaker (150 RPM). This experimental protocol was used for all further experiments described in this report (i.e., data in Table 6-21).

The method of TCE analysis was also modified. TCE was monitored by direct extraction of the culture medium and gas chromatographic analysis of the extract. A 1.5 mℓ sample of the culture medium was taken by syringe at initiation and termination of experiments and injected into a 4 mℓ vial sealed with a Teflon®-faced neoprene rubber septum and containing 1.5 mℓ pentane. The contents were then mixed thoroughly to extract TCE from the aqueous phase.

A 1.5 µℓ sample of the pentane fraction was then injected into the gas chromatograph for analysis. All gas chromatographic parameters were as described in Table 2, except the oven temperature which was 50°C. Losses of TCE from sterile bottles were greatly reduced by the use of the bottle method. However, some variability remained (recoveries from 80-95 percent) therefore, losses of TCE from nonsterile bottles were always compared to that lost from sterile controls.

3. Mineralization Studies

Measurements of $^{14}CO_2$ produced from [^{14}C]TCE were performed in test systems similar to that used for TCE metabolism experiments. [U-^{14}C]TCE was obtained from New England Nuclear Corp. The material was found to contain a radiolabelled polar contaminant (10-20 percent total ^{14}C) that remained in aqueous phase when solutions were extracted with hexane. Separation of the contaminant from the TCE was achieved by passage of aqueous solutions of the stock TCE through a Sep-Pak® C_{18} cartridge (Waters Associates, Inc.). The polar contaminant passed through the cartridge with the aqueous phase whereas the TCE remained adsorbed to the C_{18} packing. Purified [^{14}C]TCE was then readily eluted with 100 percent methanol and had a specific activity of 7 mCi/mmol. Following a 2-day incubation with radiolabeled substrate, each culture was acidified by addition of 200 µℓ of 2 M H_2SO_4 and sparged (through a syringe needle) with N_2 at a flow rate of 30 mℓ/minute for 60 minutes. The effluent gas was bubbled through two CO_2 traps in series, each containing 20 mℓ of 1 M NaOH. Radioactivity in samples of the medium and the CO_2 traps was measured by liquid scintillation counting. Presence of $^{14}CO_2$ was confirmed by precipitation of the radioactivity upon addition of $BaCl_2$. Sterilized cultures retained less than 5 percent of the added TCE, at the end of the incubation period.

4. Anaerobic Enrichments

Anaerobic media were inoculated with 10 percent (vol/vol) activated sludge obtained from the Radford, VA, municipal waste treatment plant. One enrichment contained TCE alone (50 mg/ℓ) while the other six contained one of the co-substrates, glucose, ethanol, sodium butyrate sodium succinate, sodium lactate (at 1 mM each), or CO_2 (50 percent vol/vol head space) as well as TCE. Sterile and nonsterile subcultures of each enrichment were also established.

A. RESULTS

1. Initial Enrichments

Several types of enrichment systems for the isolation of aerobic TCE degraders were designed and tested. Figure 1 shows the initial systems, designed to measure three aspects of possible TCE degradation: growth, activity, and mineralization. System A, provides continuous high concentrations of TCE in the headspace and, consequently, in the water, because of the chemical's solubility. As TCE is consumed, more can be added to the bubble tube. Five of these flasks were set up with samples from the Wurtsmith air-stripping tower and incubated for almost 6 weeks. Colonies were never observed after subsamples were plated for growth on TCE vapors. The disadvantages of the system are (1) poor facilities for adding volatile co-substrates and (2) adsorption of the TCE onto rubber stoppers.

System B is similar to A, except TCE is added once to the water phase and the system is air tight. Variability in TCE concentrations due to adsorption by the rubber stoppers, however, limited the usefulness of this system. Further modification of this system led to the use of test tubes with screw caps containing Teflon®-lined septa. This allowed TCE, other volatile co-substrates and chemicals to be injected through the septa without loss from volatilization. Subsequently, screw cap test tubes (30 mℓ) with Teflon®-lined septa were used to reduce leakage, sorption, variablity, and incubator space requirements. Changes in TCE concentration were measured in samples removed from the headspace, with a Hamilton GC gastight syringe.

A total of 43 separate enrichments were set up in the Teflon®-septum/tube system. Samples provided by the Air Force showed no TCE degradative activity despite their history of TCE exposure. Pure cultures of bacteria presumed by scientists at the Idaho National Engineering Laboratory to promote TCE degradation, were never sent to our laboratory and could not be tested. Therefore, we elected to establish a large number of additional enrichments, using samples collected from the Pensacola area.

Most of the enrichments, including the anaerobic tests, showed no loss of TCE significantly greater than losses in sterile controls. However, one of the aerobic samples (five replicates) of water from an oily holding pond at the Pensacola Naval Air Station (NAS) treatment plant showed substantial TCE disappearance in all tubes (Table 3) as compared to sterile controls. Subsequent subcultures of the sample from the NAS treatment plant did not show utilization of TCE. The activity was reestablished by use of one of the original enrichments as an inoculum with

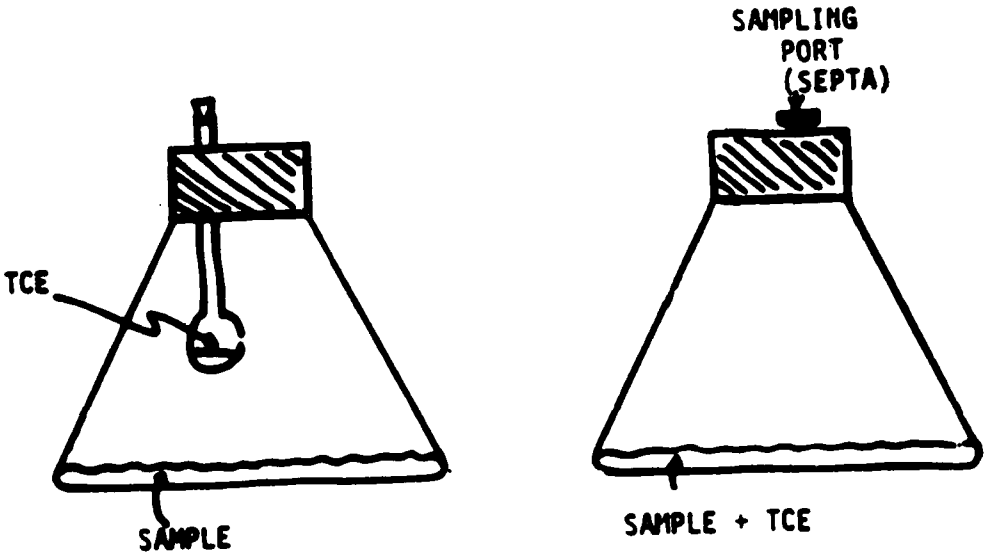

Figure 1. Laboratory Systems Used for the Enrichment of TCE-Degrading Microorganisms

TABLE 3. DISAPPEARANCE OF TCE FROM THE NAS ENRICHMENT

Sample	Percentage TCE Remaining[a]
Active 1	6.4
Active 2	45.8
Active 3	5.2
Active 4	8.7
Active 5	0
Sterile Control	100

[a] Relative to sterile controls

TABLE 4. DISAPPEARANCE OF TCE IN SERIAL SUBCULTURES OF THE NAS ENRICHMENT

Sequential Subculture[a]	Incubation Time (Days)	Percentage TCE Remaining[b]
1	2	29.0
2	1	13.8
3	1	38.9

[a] 1 mℓ of the original enrichment (for subculture No. 1) or the preced subculture was used to inoculate 5 mℓ of NAS water to which a mineral salts concentration was added to provide inorganic nutrients equivalent to MSB.

[b] Relative to sterile controls which retained at least 75 percent or greater of the added TCE.

a sample of water from the initial site of isolation to make up the media. Three sequential sets of subcultures degraded TCE when the NAS water was used for medium preparation (NAS medium) (Table 4). Subcultures without NAS water did not degrade TCE (Table 5). This suggested that some component in the water was necessary for the apparent biodegradation. The sterile controls indicated that biological activity was necessary for TCE disappearance. Yeast extract increased the rate of TCE disappearance but would not substitute for NAS water (Table 5).

TABLE 5. TCE DISAPPEARANCE IN SUBCULTURES WITH NAS WATER AND DISTILLED WATER

Medium Used For Subculture[a]	Percentage TCE Remaining[b] Days of Incubation 0	1	2	3
NAS water only	81	39	14	14
NAS water + .05% yeast extract	128	22	2.2	0.02
Distilled water + .05% yeast extract	120	109	84	96

[a] Each medium contained mineral salts equivalent to MSB.

[b] Relative to sterile controls which retained at least 75 percent or greater of the added TCE.

2. Isolation of a Pure Culture that Metabolizes TCE

The microorganisms in the TCE-degrading sample from the NAS treatment plant could be grown to high cell density in 10 mM glucose MSB and the resulting mixed culture would still metabolize TCE upon transfer into a NAS medium. We isolated an organism that metabolized TCE from the NAS sample by streaking aliquots onto glucose agar plates. Five colony types were selected and grown on slants. The cells were washed from the slants and tested for the ability to metabolize TCE by inoculation into NAS medium. Only one of the isolates caused significant TCE disappearance, as compared to controls (Table 6). The active isolate, designated G4, was serially transferred three times into NAS medium and found to promote substantial TCE disappearance each time. Strain G4 is a nonmotile gram negative bacillus which is oxidase negative, catalase positive, nonfermentative and reduces nitrate. This strain forms raised, circular, opaque colonies on glucose plates.

3. Requirements for TCE Metabolism by the Pure Culture

NAS water was required for complete TCE metabolism by strain G4 (Table 7). In some experiments, TCE also decreased (40 percent loss) in the absence of NAS water, indicating that limited TCE metabolism occurred in its absence. However, in other experiments, no TCE degradation was observed when NAS water was omitted. Addition of chloramphenicol to the medium containing NAS water prevented extensive TCE metabolism, suggesting need for protein (enzyme) synthesis for degradation to occur. Oxygen was also required for TCE metabolism, as shown by the absence of metabolism

TABLE 6. TCE DISAPPEARANCE IN CULTURES OF ISOLATES OBTAINED FROM THE NAS ENRICHMENT

	PERCENTAGE TCE REMAINING			
	1 day		4 days	
Isolate	Tube 1	Tube 2	Tube 1	Tube 2
Uninoculated	110	116	85	ND[a]
G1	130	127	108	95
G2	95	98	87	91
G3	103	92	71	54
G4	4	1	ND	ND
G5	117	112	86	87

[a] Not determined

TABLE 7. REQUIREMENT OF NAS WATER FOR TCE METABOLISM BY STRAIN G4 AND INHIBITION BY CHLORAMPHENICOL

Medium Supplement	Inoculum[b]	TCE Concentration (μM)[c] Initial	Final	Percentage Remaining
NAS water[a]	Active	1.35 ± 0.49	[d]<0.02	<1.5
NAS water	Sterilized	1.28 ± 0.35	1.42 ± 0.32	110.9
NAS Water, chloramphenicol (0.05 mg/mℓ)	Active	1.22 ± 0.20	0.91 ± 0.12	75.0
None	Active	1.22 ± 0.18	0.84 ± 0.10	68.8

[a] NAS water replaced distilled water in the medium.

[b] A stationary-phase culture of strain G4 (1 mℓ) grown on glucose medium was used as the inoculum. The inoculum was sterilized by autoclaving where indicated. Experiments were terminated after 24 hours incubation.

[c] Data are the means and standard deviations of three replicates.

[d] Minimum detectable level.

in bottles sparged with N_2 (Table 8). Controls sparged with an identical amount of air showed full activity.

TABLE 8. EFFECT OF O_2 REMOVAL ON TCE METABOLISM BY STRAIN G4

Experimental Condition[a]	Inoculum[b]	TCE Concentrations (μM)[c] Initial	Final	Percentage Remaining
Unsparged, air headspace	Active	0.99 ± 0.10	[d]<0.02	<2
Unsparged, air headspace	Sterilized	0.92 ± 0.03	0.81 ±0.01	88.0
N_2 sparged	Active	1.06 ± 0.32	0.86 ±0.32	81.1
Air sparged	Active	1.14 ± 0.01	<0.02	<1.8

[a] All experiments employed medium made with NAS water. N_2 and air sparging was performed for 30 minutes at 30 mℓ/min prior to addition of TCE and inoculum.

[b] Inoculum as in Table 7.

[c] Data are the means and standard deviations of three replicates. Experiments were terminated after 24 hours incubation.

[d] Minimum detectable level.

TCE degradation was confirmed by mineralization studies. In active samples, 60 percent of the ^{14}C remaining after incubation was converted to CO_2, whereas no formation of $^{14}CO_2$ was detected in sterile controls (Table 9). Another 35 percent of the ^{14}C remaining in the active samples was converted to unidentified, nonvolatile products, but only 3 percent of the ^{14}C present in the sterile controls remained in the nonvolatile fraction (Table 9). Four to 6 percent of the ^{14}C remaining in the active samples was present as TCE (determined by gas chromatographic analysis) and was lost during sparging to remove $^{14}CO_2$. Passage of the medium from active samples through a 0.45 μm filter removed 57-78 percent of the nonvolatile ^{14}C. Association of the radioactivity with particulate material suggests that the carbon was incorporated into cellular material. Less ^{14}C remained in the sterile samples (53 percent) than in the active samples (89 percent) after the incubation. This was most likely a result of TCE leakage or adsorption to the septa; these losses would occur throughout the incubation in the sterile samples but would be decreased in active samples because of the conversion of TCE to less volatile products.

4. Growth Substrates and Inducers of TCE Metabolism

Strain G4 grew on a variety of substrates (Table 10). However, no growth was observed with methane or methanol. TCE metabolism did not

TABLE 9. DISTRIBUTION OF RADIOACTIVITY FOLLOWING METABOLISM OF ^{14}C-TCE BY STRAIN G4[a]

Strain G4	Nonvolatile Products[c]		Carbon Dioxide	
Inoculum	DPM	Percent Total[b]	DPM	Percent Total
Active	31,445 ± 1,869	34.7 ± 1.5	53,996 ± 220	59.6 ± 1.0
Sterilized	1,785 ± 204	3.4 ± 0.5	0	0

[a] Medium was made with NAS water to which [^{14}C]TCE (101,970 DPM) and unlabeled TCE was added to give a total of 37 nmol. Each experiment was inoculated as in Table 7. Data are the means and standard deviations of three replicates.

[b] Based on total ^{14}C present after incubation as 100 percent: Active = 90,552 ± 1,759 DPM; Sterilized = 53,618 ± 2,329 DPM.

[c] Radioactivity remaining in solution following acidification and sparging to remove CO_2.

occur when the potential growth substrates at the concentrations listed in Table 10 were substituted for NAS water. Analysis of NAS water by gas chromatography (flame ionization detector) showed that some organic compound present in NAS water disappeared during TCE metabolism by Strain G4 (Figure 2). GC-mass spectroscopy of NAS water extracts (performed by Dr. William Mahaffey, University of Texas) revealed that the peak which disappeared from NAS water concurrently with TCE metabolism, was phenol. Subsequent testing confirmed that phenol would induce TCE metabolism (Table 11), and, that toluene was equally effective. Tests at higher (200 nmol) concentrations of TCE indicated o- and m-cresol were also effective inducers of TCE metabolism. All compounds that induced TCE metabolism were also growth substrates for G4. Aromatic compounds that did not induce TCE metabolism were 1, 3, 5-trimethylbenzene (Table 11), m-xylene, sodium benzoate, p-cresol (Table 12), 4-chlorobenzoate, o-, m- and p-chlorophenols, and all dichloroethylene isomers (data not shown).

TABLE 10. COMPOUNDS TESTED AS GROWTH SUBSTRATES FOR STRAIN G4[a]

Substrate	Growth[b]	TCE Degradation
Glucose	+	-
Sodium Acetate	+	-
Sodium Succinate	+	-
Sodium Pyruvate	+	-
Sodium Benzoate	+	-
Ethanol	+	-
Sodium Lactate	+	-
Sodium Butyrate	+	-
Methanol	-	-
Ethylene	-	-
Methane	-	-

[a] Each sample contained 10 mℓ of basal medium containing 0.01 percent yeast extract and the indicated potential substrate to which 0.1 mℓ of a stationary phase culture of strain G4 grown on glucose was added. After 7 days of incubation at 26°C with shaking, the optical density of each was determined at 600 nm and compared with a control sample prepared and inoculated in an identical manner, but without growth substrate. All substrates were tested at 10 mM, except ethylene and methane which were present as 50 percent (vol/vol) of the headspace and all experiments, including controls were performed in triplicate.

[b] All positive (+) cultures were > 0.8 O.D. units of the control samples and all negative (-) cultures were within 0.05 O.D. units of the control samples.

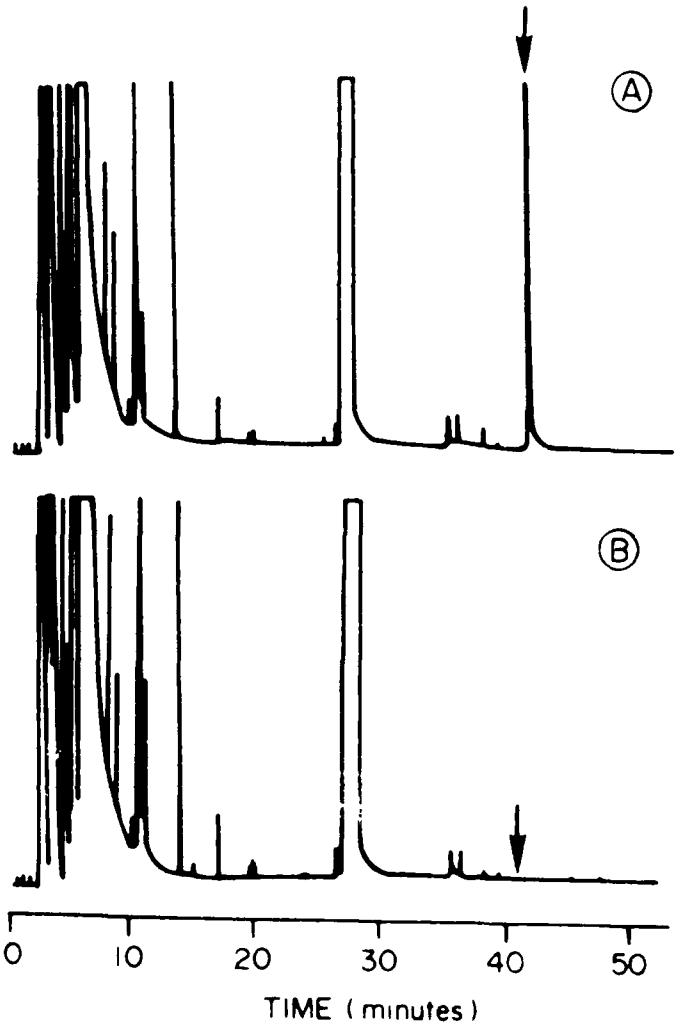

Figure 2. NAS Water Before (A) and After (B) its use as Medium for TCE Metabolism by Strain G4. The peak of interest is indicated by the arrow. Ten mℓ of each sample was extracted twice within 5 mℓ of diethylether. The combined ether extracts were then concentrated under a stream of nitrogen to a final volume of 0.75 mℓ and 1.5 µℓ was injected into the gas chromatograph. The column was a 30 m SpB1 (Supelco) capillary column. The column temperature was initially 20°C for 2 minutes followed by a linear temperature gradient of 2°C per minute to 250°C.

TABLE 11. TEST OF SEVERAL HYDROCARBONS AS INDUCERS OF TCE METABOLISM[a]

Inducer Compound	TCE Concentrations[b] Initial (μM)	Final (μM)	Percentage Remaining
NAS water	1.35 (±0.49)	[c]<0.02	<1.5
n-Decane	1.10 (±0.07)	1.01 (±0.07)	91.8
1,3,5-Trimethyl-benzene	1.20 (±0.11)	1.03 (±0.11)	85.8
Toluene	1.17 (±0.23)	<0.02	<1.7
Phenol	0.70 (±0.03)	<0.02	<2.9

[a] The indicated compounds were 1 mM (NAS water was used undiluted to make up the basal media).

[b] Data are the mean and standard deviation of three replicates.

[c] Minimum detectable level.

TABLE 12. COMPOUNDS TESTED FOR STIMULATION OF TCE METABOLISM BY STRAIN G4[a].

Compound Added	TCE Remaining[b] (μM)
None	3.35 ± 0.26
Phenol	0.04 ± 0.0
Toluene	[c]<0.02
m-Xylene	3.92 ± 0.08
Sodium benzoate	4.17 ± 0.29
o-Cresol	< 0.02
m-Cresol	0.07 ± 0.09
p-Cresol	4.04 ± 0.45

[a] Each bottle contained 200 nmol of TCE and 10 μmol of the indicated compounds. The inoculum was 1 mℓ of a late log-phase culture of strain G4 grown on 20 mM sodium lactate.

[b] A control set containing no compound and inoculated with sterilized G4 contained 3.4 ± 0.07 μM TCE after 24 hours incubation at which time the test was terminated.

[c] Minimum detectable level.

SECTION III

CHARACTERIZATION OF TCE DEGRADATION BY STRAIN G4

A. METHODS

1. Organisms and Culture Conditions.

Beijerinkia sp., Pseudomonas putida F1, and its mutants Pp39D and Pp106, were obtained from D.T. Gibson (Reference 9). P. putida strain B5 and P. putida strain mt-2 were obtained from P. Chapman. Strain G4 was isolated in our laboratory as described in Section II.B.2. All media contained basal salts (MSB, Table 1) and cultures were grown at 30°C with shaking. Cultures were maintained on 10 mM glucose medium and stored at 4°C.

2. TCE Metabolism Experiments

TCE metabolism experiments were performed as described in Section II.A.2 in 50 mℓ Wheaton vials containing 10 mℓ of MSB. Potassium phosphate buffer, 0.1 M at pH 7.0 (P7 buffer), replaced MSB in chloride production experiments. Each vial contained 200 nmol TCE and 1 mM phenol. Experiments were terminated after 24 hour incubation at 26°C, except where indicated. Data presented are the means and standard deviations of three replicate experiments. TCE analysis by gas chromarography was described in section II.A.2.

Tests for TCE metabolism in environmental samples were performed as above, except that the water sample was substituted for MSB. Potassium/sodium phosphate buffer (0.08 M) at pH 7.0 and ammonium sulfate (0.02 M) were added to the water samples. The incubation period for these tests was 4 days and phenol was added only to samples where indicated.

3. Chloride Production

Cultures of strain G4 were grown 16 hours on 20 mM sodium lactate with 2 mM phenol added as the inducer. An additional 2 mM phenol was then added and incubation was continued for 4 hours. Cells were harvested by centrifugation, resuspended in MSB to 1/20 the original volume, and 0.1 ml (ca. 7 by 10^9 colony forming units) was used as inoculum for the experiments. Each experiment was performed as previously described for TCE metabolis (III.A.2) except that P7 buffer replaced MSB. Chloride was determined with an Orion (Orion Research, Inc.) 94-17B chloride specific electrode and 90-02 reference electrode calibrated with KCl standards made up in P7 buffer. Chloride produced from TCE was calculated as chloride present in samples containing TCE (200 nmol) minus chloride present in samples with no TCE. Rate experiments were performed in replicate TCE metabolism bottles that were sacrificed for analysis at the indicated times. Tests for chloride production from other chloroaliphathics were performed as for TCE except that the test compound was substituted in an amount equal to 600 nmol of chloride equivalents.

4. Tests for the Catechol Ortho Ring-Fission Pathway

The production of β-ketoadipate from catechol was determined by the Rothera method (References 10, 11) to test for the presence of a catechol ortho ring-fission pathway. Cultures of strain G4 were grown with 2 mM phenol, toluene or benzoate and concentrated as indicated for chloride production experiments (III.A.3). A 0.2 mℓ sample of each concentrated cell suspension was added to 2.0 mℓ of 20 mM Tris-HCl buffer, pH 8.0, and 3 drops of toluene were added to solubilize cell membranes. Catechol (0.2 mℓ of a 0.1 M solution) was added as the ring-fission substrate. After 25 minutes incubation at 30°C, about 3 g of ammonium sulfate and 3 drops of 5 percent sodium nitroprusside were added. The resulting mixture was then overlaid with 1 mℓ concentrated ammonium hydroxide. A positive test for ortho-cleavage is the production of a purple band at the interface of the layers.

5. Oxidation of Aromatic Compounds.

Polarographic experiments were performed with a Gilson oxygraph (Gilson Corp.) equipped with a Clark-type electrode. Cells were grown and concentrated (III.A.4) as indicated for the test for the ortho-fission pathway. Assay mixtures contained P7 buffer, cells, and 1 mM of the test compound in a total volume of 2.0 mℓ. Background respiration was measured before addition of the test compound and was subtracted from the final rate.

6. Protein Determination.

Protein was assayed by the method of Bradford (Reference 12), after cells were preincubated for 15 minutes in 0.1 M NaOH. Bovine serum albumin was used as the standard.

B. RESULTS

1. Optimization of TCE Metabolism/Induction with Pnenol

Preliminary TCE metabolism experiments with strain G4 were conducted in MSB medium containing 1 mM phenol or some other inducer. This procedure allowed for limited growth of the inoculum and induction of the enzymes necessary for TCE metabolism.

Because of interfering background, chloride release experiments (see below) could not be performed in MSB medium. Therefore, P7 buffer was substituted for MSB medium in these experiments. Although acceptable background for chloride-release measurements resulted, no TCE metabolism occurred, even when 1 mM phenol was included. Experiments were then undertaken to optimize TCE degradation in P7 buffer. Attempts were first made to utilize phenol as the growth substrate. An initial experiment (Table 13) indicated that phenol, at 5 mM or higher concentrations was inhibitory to growth. Therefore, G4 was grown for 4 days with an initial concentration of 3 mM phenol and equivalent amounts of phenol were added on the second and third days of incubation. The culture thus obtained had lower optical density at 600 nm (OD600 = 0.77) than overnight cultures grown on glucose (OD600 = 1.2 - 1.4) and was not capable of TCE metabolism in experiments conducted in P7 buffer with or without added phenol.

TABLE 13. GROWTH OF STRAIN G4 WITH VARIOUS CONCENTRATIONS OF PHENOL[a]

Phenol Concentration (mM)	Optical Density (600 nm)	
	Initial	24 hours
1	0.25	0.40
	0.23	0.43
2	0.24	0.56
	0.25	0.52
5	0.25	0.18
	0.25	0.19
10	0.26	0.15
	0.23	0.15

[a] A 10 percent inoculum of stationary-phase strain G4 grown on glucose was added to 10 mℓ of basal medium containing the indicated concentrations of phenol. Incubation was at 30°C on a shaker. Data are for duplicate tubes.

Therefore, we grew cultures on an alternate primary carbon source with low concentrations of phenol added for induction of the aromatic pathway. Strain G4 was grown overnight on 10 mM glucose, harvested by centrifugation, resuspended in MSB containing 3 mM phenol, and incubated (30°C, with shaking) an additional 12 hours. These cells also did not metabolize TCE in P7 buffer. To test the hypothesis that optimum ability to metabolize TCE would coincide with optimum ability to metabolize phenol, an experiment was performed to determine when the enzymes for phenol oxidation were optimally induced in glucose grown cells (Figure 3). The results indicate that enzyme activities were optimally induced 6 hours after the addition of phenol to the culture. TCE was metabolized in P7 buffer when cells were grown and induced by this method (Table 14). However, phenol addition to the medium in TCE metabolism tests was still required for substantial TCE metabolism; i.e., preinduction with phenol was not sufficient for complete TCE metabolism.

We examined the effect of phenol addition at the time of inoculation and again after overnight growth. In these experiments the ability to oxidize both phenol and catechol was monitored at periods after the readdition of phenol. Lactate and glucose were the best growth substrates under the conditions tested (Figure 4). Optimum induction of both phenol and catechol oxidation occured 4 hours after the readdition of phenol to the overnight cultures.

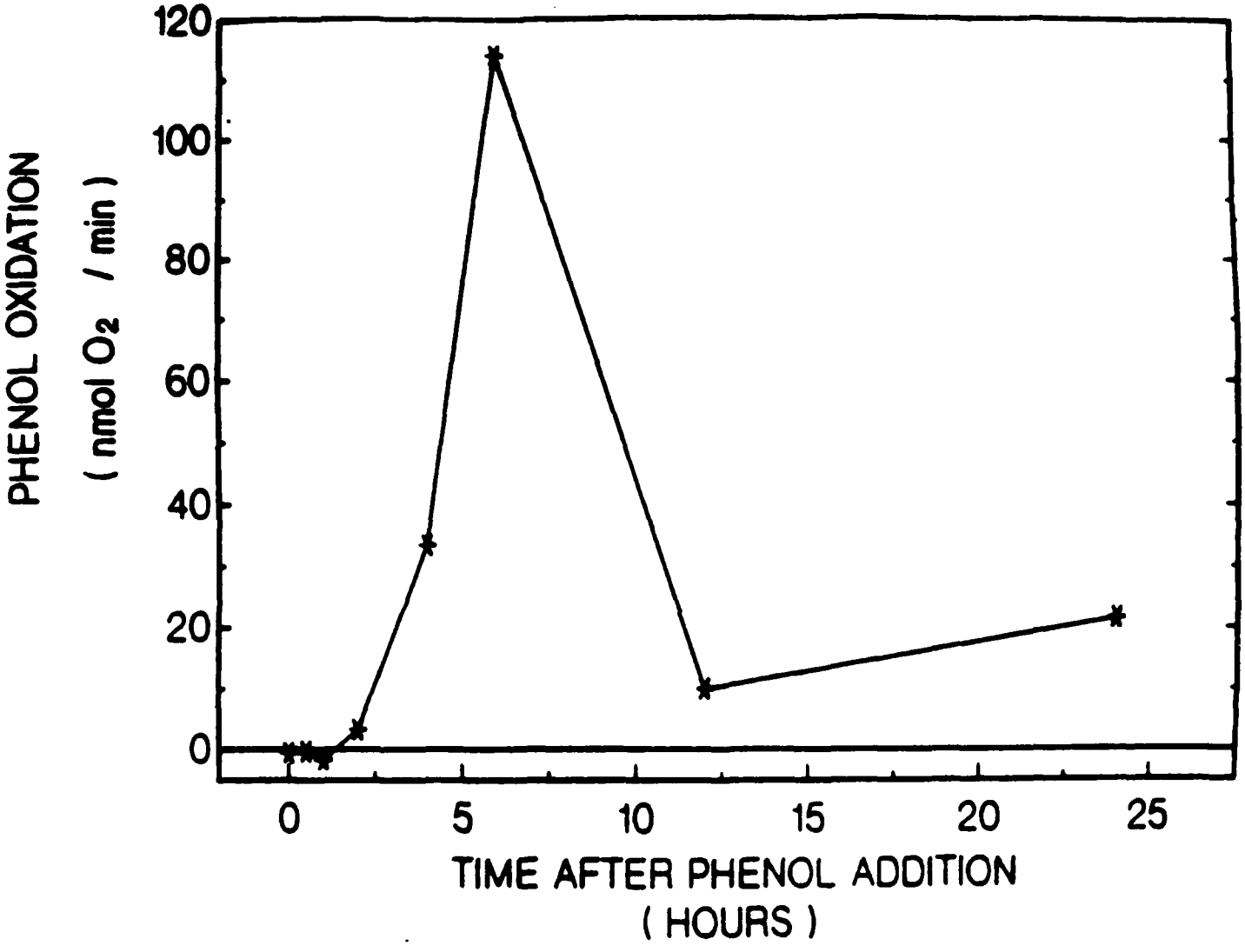

Figure 3. Time Course for Maximal Induction of Phenol Metabolism by Strain G4. Phenol (2 mM) was added to a glucose-grown (10 mM, 16 hours) culture of strain G4 and at the indicated time cells were harvested by centrifugation, resuspended in 1/20 the original volume of 0.1 M potassium phosphate buffer, pH 7.0, and 60 µℓ of the resulting cell suspension assayed for phenol oxidation in a Gilson oxygraph. Data are the mean of duplicate assays.

TABLE 14. TCE METABOLISM IN PHOSPHATE BUFFER

Medium	Inoculum[a]	Phenol Addition (1 mM)	TCE remaining[b] (μM)
Basal Medium	Sterile	+	10.6 ± 0.7
Basal Medium	Active	+	0.06 ± 0.01
Phosphate Buffer	Active	+	0.07 ± 0.03
Phosphate Buffer	Active	-	8.7 ± 0.2

[a] Strain G4 was grown for 12 hours on 10 mM glucose then 2 mM phenol was added and the culture was incubated an additional 6 hours. One mℓ samples of the resulting culture were then added as inocula to each vial. Sterile inoculum was autoclaved prior to addition. Each experiment contained 10 mℓ of the indicated medium to which 500 nmol of TCE was added. Phenol was also added as indicated.

[b] TCE was determined after 24-hour incubation. Data are the means and standard deviations of duplicate samples.

2. Chloride Release and Stoichiometry

Glucose-grown cells of strain G4, optimally induced by post growth-exposure to phenol for 6 hours, were used to determine the amount of chloride released when TCE was metabolized. Results of two separate experiments (six individual treatments) showed that 2.7 to 2.9 molecules of Cl^- were released per TCE molecule added (Table 15). The data are consistent with complete dechlorination of TCE by strain G4. The kinetics of chloride release during TCE metabolism (Figure 5) indicated a linear rate of 1.5 Cl^-/minute which corresponds to 0.50 nmol of TCE consumed/minute. Complete dechlorination (92 percent of theoretical) had occurred within 6.5 hours. This rate was compared to cells grown in lactate-phenol medium and induced by post growth exposure to phenol for 4 hours. A more rapid consumption of TCE was observed (Figure 6) and chloride was released at a rate of 3.7 Cl^-/minute or 1.3 nmol TCE consumed/minute; more than 2.5 times the rate of glucose-phenol grown cells. Complete dechlorination occurred within 3 hours.

3. Preliminary Studies on the Biodegradation Pathway for TCE

The stimulation of TCE degradation by strain G4 resulting from the addition of certain aromatic compounds to the culture medium suggested the involvement in TCE degradation of an enzyme(s) induced for degradation of aromatic compounds. Therefore, experiments were conducted to determine whether aromatic degradation pathway(s) present in strain G4 were involved in TCE degradation. An initial experiment suggested that strain G4 contained a meta ring-fission pathway for phenol degradation. When strain

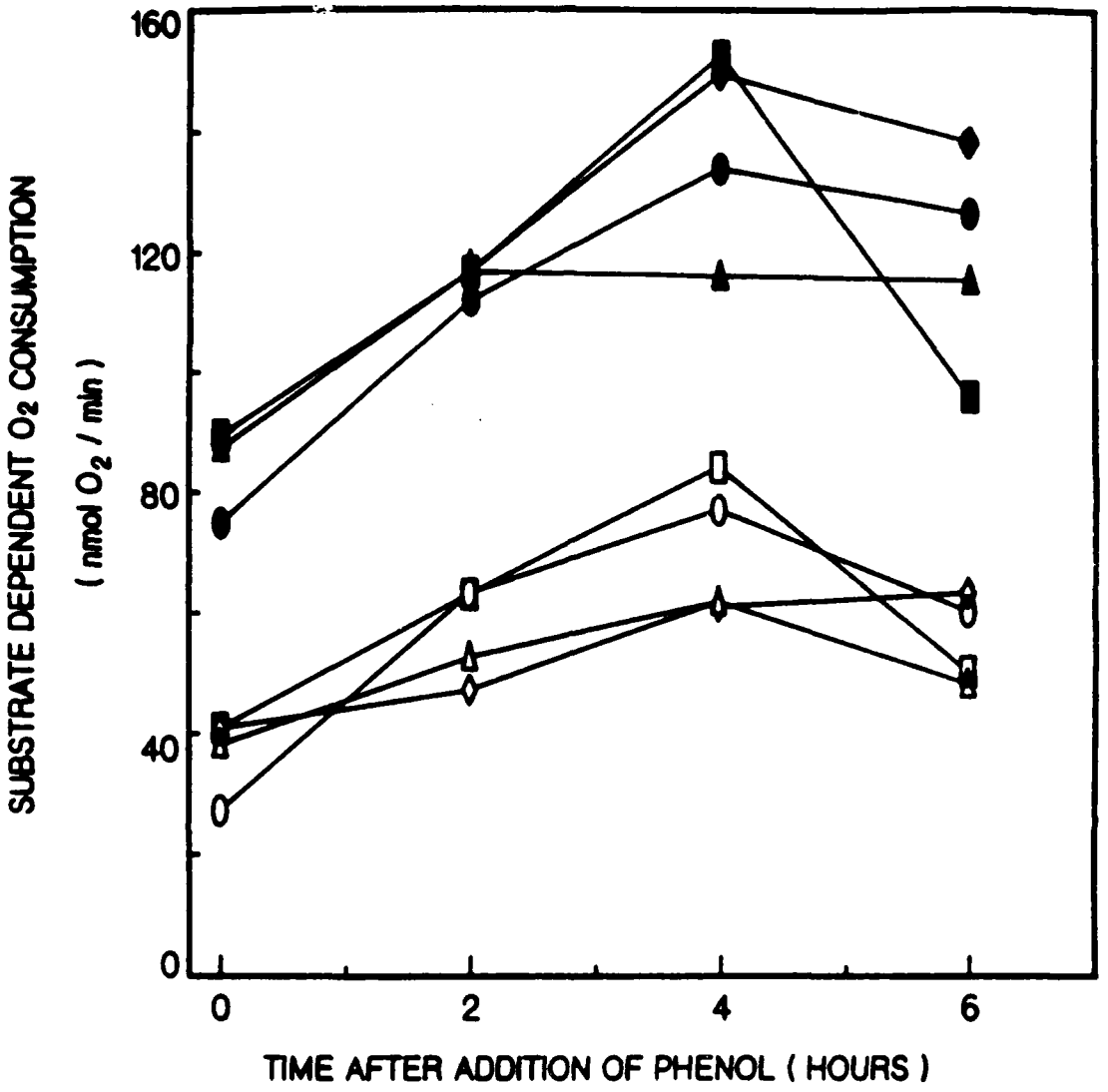

Figure 4. Effect of Growth Substrate on Phenol (Open Symbols) and Catechol (Closed Symbols) Oxidation by Cultures of Strain G4 Grown in the Presence of Phenol. Strain G4 was grown in the presence of 2 mM phenol and with one of the following growth substrates: 10 mM glucose (○, ●), 20 mM lactate, (□, ■), 15 mM succinate (△, ▲), or 30 mM acetate (◇, ◆). After overnight growth 2 mM phenol was readded to each culture, cells were harvested by centrifugation, resuspended in 0.1 M potassium phosphate, pH 7.0 to 1/20 the original volume and a 100 µℓ aliquot assayed (about 2 by 10^9 colony) forming units). Each data point is the mean of replicate assays.

TABLE 15. STOICHIOMETRY OF CHLORIDE RELEASED FROM TCE BY STRAIN G4

	Chloride Produced[a] nmol	Chloride per TCE	Percentage Theoretical[b]
Exp. 1	586 ± 34	2.93 ± 0.17	98
Exp. 2	541 ± 20	2.70 ± 0.10	90

[a] Background subtracted.

[b] Three chlorides per TCE molecule (a total of 600 nmol chloride equivalents) was used as 100 percent theoretical.

G4 was grown on agar plates with 2 mM phenol as the carbon source and subsequently sprayed with 0.1 M catechol a yellow color developed. This result is indicative of the production of the yellow meta ring-fission product, α-hydroxy-cis, cis-muconic semialdehyde by the enzyme catechol-2,3-dioxygenase (Reference 13). Growth of G4 on MSB plates with toluene vapors (added as 100 percent toluene in small test tubes stoppered with cotton and taped to the plate lid) with or without a secondary carbon source resulted in poor growth apparently due to sensitivity of strain G4 to toluene. Thus, tests for meta-cleavage were inconclusive. Growth of strain G4 overnight on agar plates with 10 mM lactate or 10 mM arginine as the carbon source followed by exposure to toluene vapors for 6 hours resulted in strong positive tests for meta ring-fission. Similar experiments in which phenol (as crystals in the plate lid or liquified phenol in test tubes) was substituted for toluene also resulted in a strong positive test for meta ring-fission. Parallel plates on which G4 was grown with either lactate, arginine or glucose alone gave negative tests for meta ring-fission. For both phenol and toluene, growth on an alternate carbon source, followed by induction with the compound of interest, resulted in stronger positive tests than growth on the aromatic compound alone. The results were probably due to production of a much larger cell mass.

The meta ring-fission test was also performed with strain G4 grown on plates containing 10 mM sodium benzoate. Sodium benzoate is an aromatic substrate that does not support TCE degradation by strain G4. No yellow color could be detected after plates were sprayed with catechol, suggesting the meta ring-fission pathway was not induced by benzoate. Cultures of strain G4 were incubated with phenol, toluene, or benzoate and tested to determine if a catechol ortho-cleavage pathway might be induced by any of these compounds. Both phenol- and toluene-induced cell suspensions turned intensely yellow with 5 minutes of catechol addition, indicative of meta-cleavage, whereas benzoate-induced cell suspensions remained colorless. Benzoate-induced cell suspensions gave a strong positive reaction for ortho-cleavage within 10 minutes of the addition of the test reagents

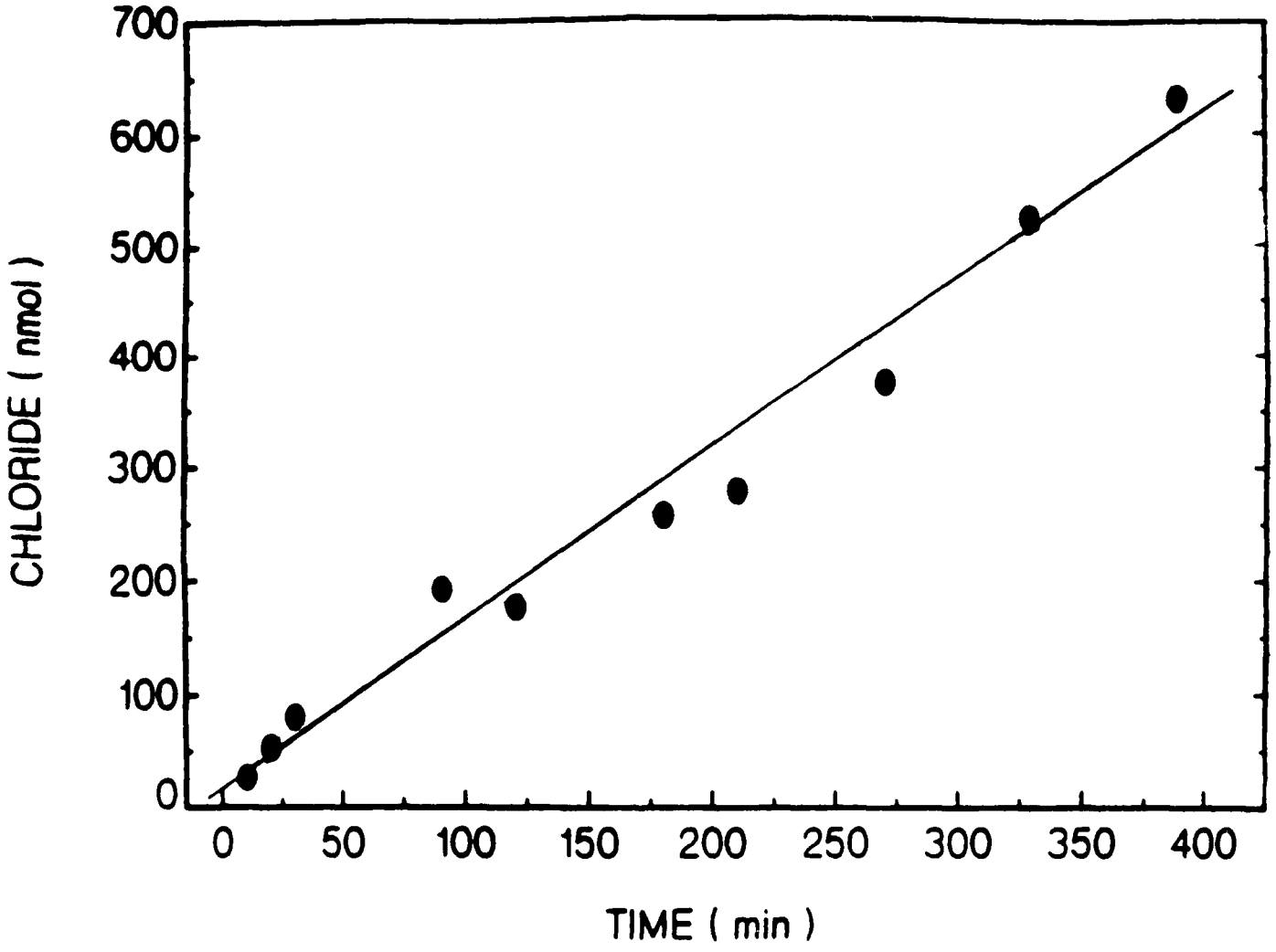

Figure 5. Kinetics of Cl^- Release from TCE by Glucose-Grown Cells with 6-Hour Postgrowth Exposure to Phenol. Each point is the mean of duplicate samples.

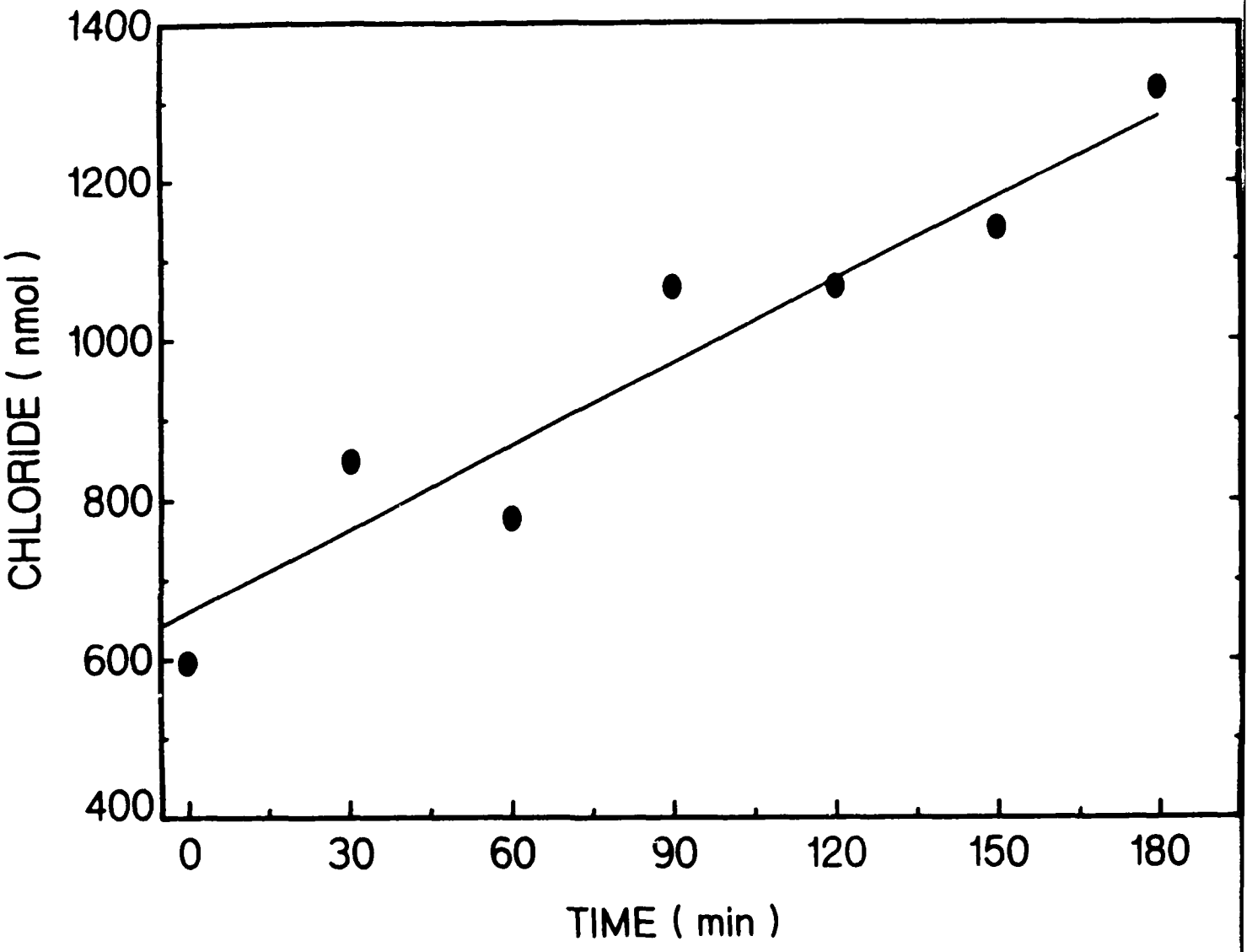

Figure 6. Kinetics of Cl^- Release From TCE by Lactate-Phenol-Grown Cells with 4-Hour Postgrowth Exposure to Phenol. Each point is the mean of duplicate samples.

nitroprusside and ammonium hydroxide. A very weak positive reaction was obtained after 15 minutes with phenol- and toluene-induced cells which indicated that some ortho-cleavage may occur under these conditions. In other experiments, when strain G4 was grown in liquid culture on benzoate, a yellow color was observed in the supernatant solution, suggesting the production of some meta ring-fission product from this substrate. However, results indicate that benzoate-induced strain G4 utilizes predominantly the ortho ring-fission pathway, whereas phenol- and toluene-induced cells utilize predominantly the meta ring-fission pathway. Thus, TCE degradative capacity appears to be associated with aromatic degradative pathways utilizing meta ring-fission.

To confirm that the yellow color observed with phenol-induced strain G4 was the meta ring-fission product, the supernatant solution of phenol-induced resting cells incubated with catechol and 3-methylcatechol was examined spectrally. The absorbance maxima of the products from both catechol and 3-methylcatechol in acid, base, and neutral conditions agreed with those published for the respective meta-cleavage products (Table 16).

TABLE 16. ABSORBANCE MAXIMA OF THE PRODUCTS OF CATECHOL AND 3-METHYLCATECHOL METABOLISM CATALYZED BY PHENOL-INDUCED STRAIN G4[a].

Substrate Added	pH	λ max Found	λ max Reported[b]
Catechol	2.5	319	320
	7.6	375.5	375
	11.0	375.5	375
3-Methylcatechol	2.5	316.5	315
	7.6	387.5	388
	11.0	387.5	388

[a] Strain G4 was grown overnight on 10 mM glucose and then induced by addition of 2 mM phenol and further incubation for 6 hours. The cells were then pelleted by centrifugation and resuspended in an equal volume of 0.05 M potassium phosphate buffer to which catechol or 3 methylcatechol (0.5 mM each) was added. After 5 minutes cells were removed by centrifugation and the supernatant solutions diluted fivefold and adjusted to the indicated pH for spectral analysis.

[b] From Reference 13.

The presence of toluene-2,3-dioxygenase in P. putida F1 results in the conversion of indole to indigo (Reference 14). Thus, active colonies of the strain become dark blue after exposure to indole vapors. Strain G4 was tested in this manner for the presence of a toluene-2,3-dioxygenase after induction with phenol or toluene. No positive test for this enzyme

was obtained with strain G4 even when strong positive tests were obtained with P. putida F1 under identical conditions. Thus, G4 does not appear to possess a toluene-2,3-dioxygenase or the enzyme it possesses is incapable of catalyzing the indigo reaction.

Polarographic studies were performed to compare the oxidation rates of strain G4 for several aromatic compounds when induced with phenol, toluene, or benzoate (Table 17).

TABLE 17. OXIDATION OF AROMATIC COMPOUNDS BY RESTING CELLS OF STRAIN G4 INDUCED WITH PHENOL, TOLUENE, OR BENZOATE.

	Substrate oxidized (nmol O_2/min/mg protein)				
Inducer[a]	Phenol	Toluene	Catechol	3-Methyl-catechol	Benzoate
Phenol	455	202	685	527	-51
Toluene	543	151	555	417	-79
Benzoate	-25	90	793	194	922
None	-21	-8.0	25	120	-28

[a] Cells were grown on lactate medium with the indicated inducers added at 2 mM (See III.A.5).

Phenol- and toluene-induced cells showed similar oxidative capabiities for the compounds tested. Phenol- or toluene-induced cells oxidized both phenol and toluene at similar respective rates, but had no benzoate-oxidizing activity. Both types of induction resulted in cells that oxidized catechol and 3-methylcatechol at similar rates (the ratio of catechol:3-methylcatechol = 1.3 for both types). Toluene- and phenol-induced cells also did not oxidize protocatechuate (data not shown). Benzoate-induced cells, however, did not oxidize toluene or phenol although the enzymes were induced for benzoate oxidation. These cells also showed distinctly different relative rates for the oxidation of catechol relative to 3-methylcatechol (catechol:3-methylcatechol = 4.1) as compared to phenol- and toluene-induced cells.

4. Transformation of Other Chloro-Aliphatics by Strain G4

Strain G4 was tested for the ability to transform a variety of chloroethanes and ethylenes based on the release of chloride from the compounds (Table 18). 1,1-Dichloroethylene and cis-1,2-dichloroethylene appeared to be transformed with the release of about one Cl^- per molecule. 1,1,2,2-Tetrachloroethane may have been partially dechlorinated but these results are not definitive due to variation in the data. All other compounds tested were apparently not dechlorinated by strain G4. The negative values obtained probably represent no dechlorination and result from variation in background chloride between active and control samples.

TABLE 18. TEST OF STRAIN G4 FOR THE ABILITY TO DECHLORINATE CHLORO-ETHANES AND ETHYLENES

Compound	Chloride Produced[a] (nmol)	Chloride Per Molecule	Percentage Theoretical
1,1-Dichloroethylene	445 ± 93	1.5	74
cis -1,2-Dichloroethylene	344 ± 153	1.2	57
trans-1,2-Dichlorethylene	67 ± 51	0.2	11
1,1-Dichloroethane	-127 ± 34	-0.4	-21
1,2-Dichloroethane	9 ± 128	0.2	1
1,1,1-Trichlorethane	-16 ± 138	-0.1	-3
1,1,2-Trichlorethane	-153 ± 26	-0.8	-26
1,1,2,2-Tetrachloroethane	89 ± 26	0.6	15
Tetrachloroethylene	-46 ± 105	-0.3	-8

[a] Background subtracted. Data are the means and standard deviations from three replicates. The compounds were added to yield 600 nmol of chloride-equivalents.

5. TCE Degradation in Environmental Samples

As a preliminary test for feasibility of TCE biodegradation in situ, experiments were performed to determine if TCE degradation could be obtained in environmental water samples (Table 19). In all cases the addition of strain G4 and phenol, with or without basal salts medium, resulted in substantial TCE degradation. The addition of toluene or phenol to the samples without G4 appeared to stimulate some metabolism of TCE by the natural flora. Preincubation of groundwater samples with phenol prior to tests for TCE metabolism resulted in a substantial increase in the ability to metabolize TCE.

6. Other Microorganisms Tested for the Ability to Metabolize TCE

The results obtained with strain G4 indicated TCE metabolism is associated with aromatic degradative capability. Therefore, several strains of bacteria capable of degrading various aromatic compounds were

TABLE 19. TCE METABOLISM IN ENVIRONMENTAL SAMPLES, WITH AND WITHOUT THE ADDITION OF STRAIN G4[a]

	TCE Remaining (μM)					
	Environmental Sample[b]					
	Estuarine		River		Groundwater	
Additions	Active	Sterile	Active	Sterile	Active	Sterile
None	2.64 ±0.13	3.05 ±0.11	3.61 ±0.31	3.93 ±0.43	3.06 ±0.67	3.58 ±0.46
Phenol, Strain G4	0.07 ±0.12	3.35 ±0.39	0.07 ±0.06	4.48 ±0.40	<0.02	2.68 ±0.46
Phenol, Strain G4 (minus Basal Salts)	0.13 ±0.12	3.16 ±0.37	0.11 ±0.09	4.92 ±0.11	<0.02	3.96 ±0.92
Toluene	2.30 ±0.35	3.15 ±0.28	1.86 ±0.02	4.38 ±0.24	2.06 ±0.81	4.59 ±0.81
Phenol	2.92 ±0.46	3.15 ±0.36	3.25 ±0.66	4.48 ±0.94	1.07 ±0.58	3.51 ±0.64
Phenol, Enrichment[c]	____	____	____	____	<0.02	____

[a]Toluene and phenol were added at 1 mM. Sterilized controls were autoclaved 15 minutes at 121° C prior to addition of TCE.

[b]All samples were water samples obtained in the vicinity of Pensacola, FL. The estuarine sample was from a pristine salt marsh on Pensacola Beach named Range Point, the river sample from the Escambia river near the University of West Florida, and the groundwater sample from an area of the Pensacola Naval Air Station known to be contaminated with jet fuel.

[c]After addition of 2 mM phenol and basal salts, samples were preincubated aerobically at 26° C with shaking for 4 days prior to addition of 10 μmol of phenol and 200 nmol of TCE to initiate TCE metabolism experiments.

tested for the ability to metabolize TCE in the presence of their respective aromatic substrates (Table 20). Under the conditions tested, only two toluene-utilizers P. putida strain B5 and strain F1 were capable of completely metabolizing TCE. These two strains degraded toluene via 3-methylcatechol (Reference 9). Another toluene-utilizer, P. putida strain mt-2, did not metabolize TCE. This organism degrades toluene via oxidation of the methyl group to from benzoate and subsequent dioxygenation to form catechol (Reference 15). Two mutants of P. putida strain F1, defective in the toluene degradative pathway, were tested for the ability to metabolize TCE (Table 21). The mutant Ppl06 lacking the first enzyme of the pathway, toluene-2,3-dioxygenase, did not show any substantial metabolism of TCE, although another mutant, Pp39D, lacking the next enzyme in the pathway, the dihydrodiol dehydrogenase, metabolized TCE as effectively as the parent strain.

TABLE 20. TCE METABOLISM BY MICROORGANISMS THAT DEGRADE AROMATIC COMPOUNDS[a]

Organism	Aromatic Substrate	TCE Remaining (μM)
P. putida NCIB 9816	Naphthalene	0.81 ± 0.06
Beijerinkia sp.	Biphenyl	0.66 ± 0.12
P. putida strain mt-2	Toluene	0.75 ± 0.17
P. putida strain B5	Toluene	<0.02
P. putida strain F1	Toluene	<0.02
None	None	0.63 ± 0.02

[a] Cultures used for inocula were grown overnight on 10 mM glucose medium and and 1 mℓ of each was used as inoculum. The indicated aromatic substrates were included in the TCE metabolism experiments at 1 mM. At initiation of the experiments, 50 nmol TCE was added and samples were incubated for 24 hours.

TABLE 21. METABOLISM OF TCE BY MUTANTS OF *P. PUTIDA* F1 UNABLE TO DEGRADE TOLUENE[a]

Strain	Defective Enzyme	TCE remaining (μM)
Parent strain	None	< 0.02
Pp 106	Toluene dioxygenase	2.98 ± 0.09
Pp 39D	Dihydrodiol dehydrogenase	< 0.02
None	-	3.84 ± 0.13

[a] Toluene at 1 mM replaced phenol in these TCE metabolism experiments.

SECTION IV

DISCUSSION

This study has established that a single microorganism can metabolize TCE to CO_2. To our knowledge, this is the first report of successful isolation of a pure culture that degrades TCE. The observed degradation may involve complete dechlorination since the radiolabeled carbon in the TCE appeared as CO_2 and seemed to be incorporated into cells. Thus, it appears that this organism is capable of complete mineralization of TCE.

Strain G4 may differ from the microorganisms responsible for TCE degradation in previous studies with natural mixed populations of bacteria. Previous studies (Reference 5) showed that natural gas was required for the aerobic degradation of TCE, suggesting that methanotrophs were involved in its degradation. Strain G4 would not grow on methane or methanol, which indicates that this organism is not a methanotroph. Furthermore, methane did not substitute for NAS water in the TCE metabolism studies. Thus, TCE metabolism by Strain G4 does not depend on the presence of a methane monooxygenase.

The requirement for NAS water suggested that some growth substrate, growth factor, or inducer was needed to trigger the synthesis of enzymes necessary for TCE metabolism. Enzyme synthesis was assumed because of the inhibitory effect of chloramphenicol. The identification of phenol toluene, o- and m-cresol as substitutes for NAS water suggested that an enzyme(s), induced for the metabolism of aromatic compounds in Strain G4, was also responsible for degradation of TCE. Two of these compounds, phenol and toluene, induce similar metabolic pathways in strain G4 that cleave the aromatic ring by meta-fission. Toluene dioxygenase, as measured by indigo formation, was not detectable in strain G4, but the enzyme in this organism may not catalyze this reaction. This enzyme should be present if toluene degradation proceeds via 3-methylcatechol (Reference 9). Toluene-induced cells readily oxidized 3-methylcatechol, but not benzoate or protocatechuate. These latter two compounds represent intermediates of the two other possible pathways for aerobic disimilation of toluene (Reference 9). Thus, strain G4 probably degrades toluene via 3-methylcatechol by utilizing a metabolic pathway for phenol and toluene degradation analogous to the toluene degradative pathway found in P. putida F1 (Reference 9). Incubation of strain G4 with either phenol or toluene produced cells that oxidized both phenol and toluene. Thus, one metabolic pathway in this organism, containing enzymes of sufficiently broad substrate specificity, may catalyze oxidation of both compounds. If this pathway is analogous to that of P. putida F1 (Reference 9), toluene would be degraded by initial dioxygenation of the 2 and 3-positions by the toluene dioxygenase forming a cis-dihydrodiol [(+)-cis-1(s),2(R)-dihydroxy-3-methylcyclohexa-3,5-diene]. This dihydrodiol would be converted to 3-methylcatechol by a specific dehydrogenase. In an analogous manner, phenol might also be degraded by dioxygenation at the 2- and 3-positiions forming its corresponding cis-dihydrodiol. However, due to the instability of this product a spontaneous dehydration would occur, resulting in the production of catechol. Thus, a monohydroxylation of phenol would result from the dioxygenase reaction. Monohydroxylated aromatic compounds are readily

formed by acid catalyzed dehydration of the dihydrdiols of toluene (Reference 16), benzoate and several alkylbenzoates (Reference 17). The conversion of indole to indigo by several aromatic dioxygenases is also apparently due to monohydroxylation of indole resulting from spontaneous dehydration of a dihydrodiol (Reference 14).

It is not clear which enzyme(s) in the aromatic degradative pathway is responsible for TCE metabolism. Oxidation of TCE by oxygenase-type reactions would be a likely mechanism for degradation. Two candidates are the enzymes responsible for initial oxidation of the aromatic ring and the catechol-2,3-dioxygenase, which cleaves the aromatic ring by meta-fission. Both of these activities appear to be absent or greatly reduced in benzoate-induced cells which do not metabolize TCE. No definitive evidence for the involvment of either the initial enzyme or the catechol-2,3-dioxygenase in TCE metabolism has been obtained to date. However, the results obtained with P. putida F1 indicate that the toluene dioxygenase of this organism is required for the degradation. Thus, the initial enzyme for ring oxidation in strain G4 may be responsible for its TCE-degrading ability.

The data obtained with the chloride specific electrode indicate all three chlorine atoms of TCE are converted to inorganic chloride by strain G4. These results are consistent with the complete mineralization of TCE. Mineralization was confirmed by production of $^{14}CO_2$ from [U-^{14}C]TCE shown in Section III. Complete degradation of TCE to nonchlorinated compounds may be the result of initial attack by either a monooxygenase or a dioxygenase forming an epoxide or dioxetane intermediate, respectively. Unstable hydroxy- or oxo-chlorinated compounds spontaneously formed from these intermediates could further decompose to form nonchlorinated compounds which could then be converted to CO_2 by enzymatic or chemical oxidations. Evidence for the formation of glyoxylate, formate and CO from decomposition of TCE epoxide has been reported (Reference 18).

SECTION V

CONCLUSIONS AND RECOMMENDATIONS

A. CONCLUSIONS

The results of this report indicate that;

1. Samples provided by the Air Force were not capable of degrading TCE under the conditions tested.

2. Naturally occurring aerobic microorganisms can degrade TCE when induced with certain aromatic compounds.

3. Biodegradation of TCE did not result from a pathway specifically for TCE metabolism; rather a pathway(s) induced for the metabolism of certain aromatic substrates was used fortuitously.

4. Aerobic biodegradation of TCE results in total dechlorination and in the production of nontoxic products.

5. Some other chlorinated compounds may also be detoxified by Strain G4. Two compounds tested: 1,1-dichloroethylene, and cis-1,2-dichloroethylene, were at least partially degraded.

6. The feasibility of biologically decontaminating a TCE-polluted environment is advanced by this work. This could be done by either adding the TCE degrader, Strain G4, to the contaminated area or by adding aromatic co-substrates to stimulate natural microbial communities to degrade TCE.

B. RECOMMENDATIONS

Based on the results, continued research is recommended in the following areas:

1. Determination of the products of degradation of other chlorinated compounds attacked by strain G4 to establish whether these compounds are also detoxified.

2. Further investigations of the aromatic metabolic pathways that are involved in TCE metabolism in both strain G4 and P. putida strain F1, to determine the enzymes involved and the mechanisms of detoxification. The information obtained can be used for developing improved strains for TCE degradation as well as strains for detoxification of other chlorinated pollutants.

3. Initiation of genetic studies to isolate and characterize the genes responsible for TCE degradation.

4. Construction of a strain that will degrade TCE without addition of aromatic co-substrates, using the information obtained under Items 2 and 3.

5. Development of a system for onsite degradation of TCE by use of a specific TCE-degrading strain or by stimulation of natural flora. Application of the system to pilot-scale cleanup of TCE at an actual contaminated site.

SECTION VI

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